Danazol induces resistance to both insulin and glucagon in young women

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INTRODUCTION

Danazol ([1,2]-oxazolo[4,5-b]-17α-pregna-2,4-dien-20-yn-17β-ol) is an impeded androgen, used in the treatment of endometriosis. Danazol causes hyperinsulinaemia [1-6], hyperglucagonaemia [1-4], a raised low-density lipoprotein (LDL) cholesterol concentration and a reduced high-density lipoprotein (HDL) cholesterol concentration [7-9]. The physiological disturbances underlying these changes are poorly understood, and, since these changes have generally been studied in isolation, it is not known whether they are related through a single underlying action of danazol, or whether they reflect independent effects of the steroid.

Computer modelling analysis of intravenous glucose tolerance test (IVGTT) glucose, insulin and C-peptide concentration profiles enables measures of insulin resistance, secretion and metabolism to be obtained using a single clinical procedure [10-12]. We have applied this approach in the present study of women receiving danazol therapy. We have also evaluated glucagon resistance using the glucagon sensitivity test (GST) and have measured fasting plasma lipid and lipoprotein levels.

This work was first presented at the 181st meeting of the Society for Endocrinology, 14-16 November 1990 [12a].

METHODS

Subjects

We studied 17 premenopausal women with laparoscopically proven endometriosis; all were less than 120% of their ideal body weight (Metropolitan Life Tables) and did not have any medical conditions or were taking any

Key words: danazol, glucagon, insulin resistance, mathematical modelling.

Abbreviations: GST, glucagon sensitivity test; HDL, high-density lipoprotein; HDL2, high-density lipoprotein subfraction 2; HDL3, high-density lipoprotein subfraction 3; IVGTT, intravenous glucose tolerance test; LDL, low-density lipoprotein.

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drugs known to affect carbohydrate or lipid metabolism. Women were recruited from the Infertility Clinic, King's College School of Medicine and Dentistry. The study received ethical committee approval at both participating institutions and all women gave their informed consent.

Tests were carried out before treatment, then after 3 months on treatment with danazol (Danol; Sterling Winthrop, Guildford, Surrey, U.K.; 400 mg daily), and finally 3 months after treatment. Women were randomly allocated on a 2:1 basis to receive either an IVGTT (11 subjects) or a GST (six subjects). Four women did not undergo tests after treatment either having become pregnant or having changed their treatment.

**Procedures**

Metabolic tests were carried out on a metabolic day ward. Patients were instructed to consume > 200 g of carbohydrate/day in their diet for the previous 3 days as preparation for their carbohydrate metabolism studies, to have fasted overnight (> 12 h) and to have taken only water and have refrained from cigarette smoking on the morning of the test. Tests commenced between 09.00 and 10.00 hours. After resting for 15 min in a semi-recumbent position, the patient had an indwelling teflon cannula inserted into an antecubital vein of each arm under local anaesthesia. Fifteen minutes later a blood sample for measurements of fasting serum lipid and lipoprotein concentrations was taken into plastic tubes without anticoagulant. Immediately after this, two samples, 5 min apart, were taken into lithium-heparin-coated tubes containing 100 μl of protease inhibitor (Trasylol; Bayer, Newbury, Berks, U.K.; 20 000 i.u./ml) for measurement of plasma glucose, insulin, C-peptide and pancreatic glucagon concentrations. The patient then received either an IVGTT or a GST.

For the IVGTT, a bolus injection of a 50% (w/v) d-glucose solution (0.5 g/kg body weight) was injected through the left antecubital vein over 3 min. Venous blood samples (7–8 ml) were taken from the contralateral cannula at 1, 2, 3, 4, 5, 7, 10, 15, 20, 30, 45, 60, 75, 90, 120, 150 and 180 min after the glucose injection for measurement of plasma glucose, insulin, C-peptide and pancreatic glucagon concentrations. For the GST an identical procedure was adopted, except that 0.5 mg of glucagon was injected instead of d-glucose.

**Laboratory procedures**

Plasma glucose concentration was measured by a glucose oxidase procedure. Hormone and peptide concentrations were measured by radioimmunoassay on samples stored at −20°C. Plasma insulin concentration was measured by using the procedure of Albano et al. [13]. Plasma C-peptide concentration was assayed by using the kit supplied by Guildhay Ltd, Guildford, Surrey, U.K., and plasma pancreatic glucagon concentration by using an antibody specific for the porcine C-terminus [14]. Cross-reactivity of danazol with the glucagon antibody was less than 0.1% in the presence of danazol at a concentration of 7.5 mmol/l. Proinsulin and 32–33 split proinsulin were measured by two-site immunoradiometric assays [15], with antibodies supplied by Serono Diagnostics, Woking, Surrey, U.K. Lipid and lipoprotein concentrations were measured in serum stored at 4°C. Serum total cholesterol and triacylglycerol concentration were measured by fully enzymic procedures [16, 17]. Concentrations of HDL and HDL subfraction 3 (HDL₃) were measured after sequential precipitation with heparin and manganese ions and dextran sulphate, respectively [18, 19]. HDL subfraction 2 (HDL₂) cholesterol was calculated as the difference between HDL and HDL₃ cholesterol. LDL cholesterol was calculated by the Friedewald equation [20]. Apolipoproteins A1, AII and B were measured by immunoturbidimetry [21]. Quality control was monitored by use of commercially available freeze-dried sera and participation in national schemes. Within- and between-batch coefficients of variation were respectively 1.5 and 1.9% (plasma glucose concentration), 3.4 and 5.8% (plasma insulin concentration, mean 3.4 μ-units/ml), 6.7 and 7.4% (plasma insulin concentration, mean 29 μ-units/ml), 3.7 and 4.0% (plasma insulin concentration, mean 133 μ-units/ml), 7.3 and 8.9% (plasma C-peptide concentration, mean 3.0 ng/ml), 7.3 and 12.4% (plasma pancreatic glucagon concentration), 0.9 and 1.3% (serum total cholesterol concentration), 1.1 and 1.9% (serum triacylglycerol concentration), 2.4 and 3.1% (serum HDL cholesterol concentration), 5.4 and 6.8% (serum HDL₂ cholesterol concentration), 6.5 and 8.1% (serum HDL₃ cholesterol concentration), 2.9 and 3.5% (serum apolipoprotein A1 concentration), 1.9 and 3.0% (serum apolipoprotein AII concentration), 2.5 and 3.1% (serum apolipoprotein B concentration).

**Modelling analyses**

Modelling analyses were carried out using programs written in FORTRAN 77 and run on a PDP 11/83 minicomputer. Glucose elimination was analysed using the minimal model of glucose disappearance of Bergman et al. [10]. According to this procedure, parameters of the equations of the model are determined which enable prediction of the IVGTT glucose concentrations from the insulin concentrations, and provide measures of the sensitivity of glucose elimination to insulin ($S_I$), inversely proportional to insulin resistance) and of glucose-dependent glucose elimination ($S_g$).

The plasma insulin concentration profile during the IVGTT reflects the first and second phases of pancreatic insulin secretion. These plasma insulin concentration changes were described according to the model of posthepatic insulin delivery of Toffolo et al. [11]. The model predicts the IVGTT insulin concentrations from the glucose concentrations, and provides a measure of the sensitivity of net first-phase post-hepatic insulin delivery to glucose ($φ_1$, first-phase insulin responsiveness), which is a function of the initial insulin and glucose responses and the insulin half-life. The model also provides measures of the sensitivity of second-phase post-hepatic insulin delivery to glucose ($φ_2$, second-phase insulin responsive-
ness) and the insulin elimination constant \( (k_i = \ln 2/\text{insulin half-life}) \). Since approximately 50% of insulin secreted by the pancreas is taken up by the liver before entering the general circulation, this model applies only to post-hepatic insulin delivery.

Pancreatic insulin secretion was analysed by the model of Vølund et al. [22], using the model identification system of Watanabe et al. [12]. The model uses insulin and C-peptide concentrations during the IVGTT. C-peptide is secreted simultaneously and in equimolar quantities with insulin, but is not subject to hepatic uptake. C-peptide measurements thus enable true pancreatic insulin secretion to be assessed [23]. Parameters of the model equations are determined which enable simultaneous prediction of both insulin and C-peptide concentrations during the IVGTT. These parameters include the hepatic insulin throughput index \( (f) \), elimination constants for insulin \( (k_i = \ln 2/\text{insulin half-life}) \) and C-peptide \( (k_c = \ln 2/\text{C-peptide half-life}) \) and pancreatic insulin secretion rates in the basal state before the IVGTT and at each sampling time during the test. From these secretion rates, net basal insulin secretion and the net increment in pancreatic insulin secretion above basal during the IVGTT can be derived. Analysis using this model distinguishes the first and second phases of pancreatic insulin secretion, enabling net incremental first- \((IS_1)\) and second \((IS_2)\)-phase pancreatic insulin secretion to be determined.

For a model identification to be acceptable, parameter estimates were required to have fractional SDS of less than 100% and to be positive.

A detailed description of the modelling analyses used in this study has been deposited as Clinical Science Appendix 92/1 with the Librarian, Royal Society of Medicine, 1 Wimpole Street, London W1M 8AE, from whom copies are available on request.

**Data analyses**

Fasting plasma levels of glucose, insulin, C-peptide and glucagon were obtained as the mean of the two samples taken immediately before the IVGTT or GST. The total area under the glucose, insulin or C-peptide concentration profiles was calculated by using the trapezium rule. In the present study, we have used the incremental area (= total area – 180 \times \text{the mean fasting concentration}) under the curves, as this provides a measure of IVGTT response unconfounded by changes in the fasting level.

Statistical analyses were carried out using BMDP Statistical Software (Los Angeles, CA, U.S.A.). Median values for each measure were obtained and paired comparisons between, before, on and after treatment values were made by using Wilcoxon’s paired signed ranks test. One of the 11 patients receiving an IVGTT had only 10 samples taken during her pre-treatment test. After an evaluation of the effects on derived measures of reducing the number of samples during the IVGTT (I. F. Godsland et al., unpublished work), concentration profile areas from this test were not included in the analysis, but model-derived measures were included. Post-treatment tests were not carried out for three of the 11 patients receiving IVGTTs and for one of the six patients receiving GSTs.

**RESULTS**

Danazol reduced fasting plasma glucose and insulin concentrations by 9% and 56%, respectively (Table 1). There was a non-significant fall of 21% in the fasting C-peptide concentration. The fasting glucagon level rose six-fold. The IVGTT glucose incremental area was unchanged on treatment, whereas the incremental insulin and C-peptide areas were increased by about twofold.

| Table 1. Fasting plasma concentrations and IVGTT incremental areas for glucose, insulin, C-peptide and glucagon in women treated with danazol for 3 months. Results are medians with ranges in parentheses. Statistical significance: *P<0.05, †P<0.01 compared with before treatment. |
|---|---|---|---|
| **Fasting plasma concn.** | **Before treatment** | **On treatment** | **After treatment** |
| n | 17 | 17 | 13 |
| Glucose (mmol/l) | 4.74 (4.22-5.50) | 4.31 (4.08-4.92)† | 4.72 (4.6-6.7) |
| Insulin (pmol/ml) | 0.036 (0.007-0.127) | 0.016 (0.007-0.098)* | 0.029 (0.007-0.146) |
| C-peptide (pmol/ml) | 0.50 (0.16-0.82) | 0.40 (0.17-0.79) | 0.47 (0.25-1.02) |
| Glucagon (pmol/ml) | 0.008 (0.001-0.138) | 0.056 (0.012-0.395)† | 0.005 (0.001-0.022) |
| **IVGTT incremental area** | | | |
| n | 11 | 11 | 8 |
| Glucose (mmol min ml⁻¹) | 363 (181-655) | 356 (203-595) | 364 (230-611) |
| Insulin (pmol min ml⁻¹) | 18.6 (7.5-40.6) | 39.0 (17.8-81.0)* | 21.6 (10.9-26.8) |
| C-peptide (pmol min ml⁻¹) | 64.1 (18.9-104.6) | 113.8 (66.1-228.4)* | 95.1 (71.8-129.2)* |
| Glucagon (pmol min ml⁻¹) | -0.2 (-0.7 to +0.35) | -2.5 (-9.8 to +5.2)* | 0.01 (-3.5 to +1.1) |
Proinsulin comprised 3.5% and 2.7% of total immunoreactive insulin before treatment and on treatment, respectively. 32-33 split proinsulin comprised 5.3% and 6.2% of total immunoreactive insulin before treatment and on treatment, respectively. The net decrement in the plasma glucagon concentration during the IVGTT was increased on treatment (Table 1, Fig. 1). Glucagon concentrations both at the peak and during the initial 20 min of the GST were identical for before and on treatment tests, only diverging later in the test as the difference in basal concentrations was re-established (Fig. 2). The GST glucose response was substantially reduced on treatment (Fig. 2). Calculation of the incremental area under the glucose concentration profile indicated that there was a 52% reduction in response, and both the insulin and C-peptide incremental areas also declined (results not shown).

Insulin sensitivity, $S_i$, decreased by 55% on treatment (Table 2). First-phase post-hepatic insulin responsiveness, $g_i$, was increased almost threefold, and the insulin elimination constant, $n_i$, was reduced by 46%. This reduction in the insulin elimination rate was confirmed by the 40% reduction in the insulin elimination constant, $k_i$, derived from the pancreatic insulin secretion model. Total pancreatic insulin secretion, $I_S$, was increased by 66% and this was associated with a 76% increase in first-phase pancreatic insulin secretion, $I_{S_1}$.

Serum total cholesterol and triacylglycerol concentrations did not change with treatment (Table 3), but there was an increase of 20% in the calculated serum LDL cholesterol concentration and a decrease of 46% in the serum HDL cholesterol concentration. Serum concentrations of both the HDL cholesterol subfractions were reduced, HDL$_{3}$ by 31% and HDL$_{2}$ by 80%. The serum apolipoprotein B concentration increased by 48% and the serum apolipoprotein AI and apolipoprotein AII concentrations fell by 42% and 11%, respectively. There were no significant correlations between changes in plasma insulin concentration, insulin secretion and insulin sensitivity and changes in serum lipid and lipoprotein concentrations, with no correlations being greater than $r = 0.5$.

All values normalized within 3 months of stopping danazol treatment, except for the plasma C-peptide concentration, which fell but remained elevated compared with the pre-treatment level.

**DISCUSSION**

Our findings indicate that danazol causes marked resistance to both insulin and glucagon. Danazol-induced insulin resistance was evident in an unchanged IVGTT glucose concentration response in association with a greatly elevated insulin response, and was confirmed by measurement of insulin sensitivity. Glucagon resistance was apparent in the greatly increased fasting plasma glucagon concentration in the absence of any elevation in fasting plasma glucose concentration, and was confirmed by the GST. Insulin resistance was manifest in the glucose-stimulated state, whereas glucagon resistance was apparent in the fasted state, as would be expected from the respective areas of action of these two hormones. The question arises: are these states of resistance related?

Whilst little is known of the nature of glucagon resistance, insulin resistance has been investigated in some detail [24]. Insulin dynamics in states of insulin resistance are determined by the mutual feedback between glucose and insulin. In insulin-resistant subjects, insulin released from the pancreas in the first-phase response to glucose is less effective than normal in promoting glucose disposal. Because of this, there is additional glucose remaining in the plasma. This acts as an additional stimulus to pancreatic insulin secretion and plasma insulin concentrations rise to normalize the rate of glucose disposal. Thus,
when there is insulin resistance, there is a prolonged and augmented second phase of insulin release. This is not the pattern seen with danazol. Instead, the greatest increase in insulin is seen in the first phase. First-phase plasma insulin responsiveness to glucose, $\phi_1$, was increased substantially, and first-phase pancreatic insulin secretion, $IS_1$, was also greatly increased, despite there being no increase in the plasma glucose concentration during the test. These findings suggest that danazol has a primary effect on the sensitivity of pancreatic insulin secretion to glucose.

Given the close association between insulin and glucagon secretion, it is possible that danazol may also have a primary effect on the sensitivity of glucagon secretion to stimuli. Steroid hormones can induce pancreatic hypertrophy and hyperplasia, leading to an exaggerated response to glucose, as in the case of insulin [25-27]. If there is indeed an exaggerated glucagon and insulin response by the pancreas to stimuli, the resistance to these hormones that we have shown could then be explained by the widespread phenomenon of receptor down-regulation in response to the elevated hormone levels [28, 29]. The reduced rate of insulin elimination in women taking danazol, as shown by the changes in parameters $n_1$ and $k_1$, is consistent with such down-regulation, since insulin elimination appears to be a receptor-mediated process [30] and is reduced in insulin resistance [31].

A possible explanation for the hyperinsulinaemia associated with danazol treatment might be that there is disrupted processing of proinsulin to insulin and C-peptide, with an increased proportion of proinsulin and 32–33 split proinsulin relative to insulin entering the circulation [32]. However, we did not find an increased proportion of insulin propeptides in women taking danazol. Disruption of the normal pulsatile pattern of insulin secretion might also contribute to the apparent increase in insulin resistance on danazol treatment [33], and might also contribute to the glucagon resistance, since pulsatile secretion of the two hormones is synchronous [34].

Insulin resistance is generally associated with fasting hyperinsulinaemia [35], whereas in the present study a reduction in the fasting plasma insulin concentration was
observed. However, the normal association between insulin resistance and the fasting plasma insulin concentration could be modified by glucagon resistance. Glucagon resistance could explain the observed reduction in the fasting plasma glucose concentration, since glucagon would be expected to be less effective in maintaining the fasting plasma glucose concentration. The reduction in the plasma insulin concentration would then be secondary to the fall in the plasma glucose concentration. The anabolic effects of danazol could also contribute. We have observed a 16% increase in lean tissue mass in women taking danazol [35a], representing a substantial increase in glucose-metabolizing tissue. It is interesting to note that glucose-dependent glucose disposal, $S_p$, increased by 47%. Although this was not significant ($P = 0.09$), such a change would be consistent with an increase in lean tissue mass.

Insulin is an important regulator of lipid metabolism. Contrary to our expectations, the effects of hypersecretion of insulin did not extend to the changes in serum lipoprotein concentrations, since the changes in insulin-related measures induced by danazol did not correlate with changes in serum lipoprotein concentrations. This suggests that danazol had independent effects on carbohydrate and lipid metabolism.

The overall effect of danazol was to induce changes in three aspects of metabolism, which are also seen in subjects at increased risk of cardiovascular disease: hyperinsulinaemia [36, 37], an elevated serum LDL cholesterol concentration [38] and a reduced serum HDL cholesterol concentration [39]. These changes commend caution in the long-term use of danazol.

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